TALITA SIGNORETI GRAZIANO

EFFECTS OF STATINS ON THE BACTERIAL VIABILITY AND ON BIOFILM OF *STAPHYLOCOCCUS AUREUS*

EFEITOS DAS ESTATINAS SOBRE A VIABILIDADE BACTERIANA E SOBRE O BIOFILME DE *STAPHYLOCOCCUS AUREUS*

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Universidade Estadual de Campinas

Faculdade de Odontologia de Piracicaba

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Dissertation presented to the Piracicaba Dental School of the University of Campinas in partial fulfillment of the requirements for the degree of Master Dentistry, in the Pharmacology, Anesthesiology and Therapeutic area.

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Co-orientador: Prof. Dr. Francisco Carlos Groppo

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ABSTRACT

Statins are drugs that competitively inhibit the enzyme 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA). Besides their important lipid-lowering action, they also are pleiotropic agents. Several studies have explored a possible protective effect of statins to reduce the morbidity and mortality of various infectious diseases. The antimicrobial activity of statins has been reported by *in vivo* and *in vitro* studies. The aim of this study was to evaluate the effects of statins on the growth, viability and biofilm formation of pathogenic aerobic bacteria. The Minimum Inhibitory Concentrations (MIC) of atorvastatin, pravastatin and simvastatin against planktonic cells of *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli* and *Enterococcus faecalis* strains were obtained. Since simvastatin showed activity against *S. aureus*, its effects on cell viability were evaluated in a time-kill and post-antibiotic effect (PAE) assays. A possible synergistic effect between simvastatin and vancomycin was also assessed. In addition, the effect of simvastatin against biofilms of *S. aureus* was tested. The MIC values of simvastatin for *S. aureus* were: 15.65 µg/ml (ATCC 29213) and 31.25 µg/ml (ATCC 33591, 43300, 14458 and 6538). The effect showed by simvastatin was dose-dependent, with a bactericidal effect at 4x > MIC concentrations and a bacteriostatic effect at MIC concentration. No synergistic effect was found between simvastatin and vancomycin. Simvastatin was able to reduce the formation of biofilms in concentrations ranging from $^{1}/_{8}$MIC to 4xMIC. In addition, the 4xMIC was able to decrease the viability, biomass and production of extracellular polysaccharides and increase the production of intracellular polysaccharides on mature biofilm of *S. aureus*. The protein production on biofilm was not altered in the presence of simvastatin . In conclusion, our results showed that simvastatin has a great potential to be explored, especially in relation to the development new antimicrobial agents.

Key-words: Statins. *Staphylococcus aureus*. Biofilms.

RESUMO

As estatinas são um grupo de fármacos que atuam como inibidores competitivos da enzima 3-Hidroxi-3-MetilGlutaril Coenzima-A Redutase (HMG-CoA redutase). Além de importantes agentes hipolipemiantes, também apresentam outros efeitos, chamados de pleiotrópicos. Diversos estudos têm explorado um possível efeito protetor das estatinas atuando na redução na morbidade e mortalidade de várias doenças infecciosas. A atividade antimicrobiana das estatinas tem sido reportada por estudos *in vivo* e *in vitro*. O objetivo desse estudo foi avaliar os efeitos das estatinas sobre o crescimento e viabilidade de bactérias aeróbias patogênicas, e o efeito da sinvastatina sobre o biofilme de *Staphylococcus aureus*. Culturas das espécies de *Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia coli e Enterococcus faecalis* foram avaliadas na forma planctônica quanto à sensibilidade à atorvastatina, pravastatina e sinvastatina, através do teste de Concentração Inibitória Mínima (CIM). Além disso, diante da atividade apresentada pela sinvastatina contra *S. aureus*, foi determinada a ação desse fármacosobre a viabilidade celular através dos testes de *Time-kill* e Efeito pós-antibiótico (EPA). Também foi verificado um possível efeito sinérgico entre a sinvastatina e vancomicina. Por fim, a ação da sinvastatina foi avaliada contra biofilmes de *S. aureus*. Os valores de CIM da sinvastatina para o microrganismo *S. aureus* foram: 15,65 µg/ml (ATCC 29213) e 31,25 µg/ml (ATCC 33591, 43300, 14458 e 6538). O efeito apresentado pela sinvastatina foi dose-dependente, sendo de caráter bactericida para concentrações 4x > MIC e bacteriostático para a concentração igual ao MIC. Não foi encontrado nenhum tipo de interação entre a associação de sinvastatina e vancomicina. Entretanto, a sinvastatina foi capaz de reduzir a formação do biofilme nas concentrações entre 1/8CIM à 4xCIM. Além disso, na concentração 4xMIC foi capaz de diminuir a viabilidade, biomassa e a produção de polissacarídeos extracelulares e aumentar a produção de polissacarídeos intracelulares de biofilmes maduros de *S. aureus*. A produção de proteínas pelo biofilme não foi

alterada. Em conclusão, os resultados encontrados mostram que a sinvastatina possui um grande potencial a ser explorado, principalmente em relação ao descobrimento de novos antimicrobianos.

Palavras-chave: Estatinas. *Staphylococcus aureus*. Biofilmes.

SUMÁRIO

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INTRODUÇÃO

As estatinas são os agentes hipolipemiantes mais utilizado para a redução de lipídios em pacientes com níveis elevados de colesterol. Esse grupo de medicamentos apresenta boa margem de segurança e tolerância durante utilização prolongada, com baixa frequência de efeitos colaterais (Maron et al., 2000; Wilt et al., 2004).

As primeiras estatinas descobertas foram a mevastatina e lovastatina, tendo sido isoladas a partir de culturas de *Penicillium citrinum* e *Aspergillus terreus* (Alberts et al., 1980). Com o intuito de aprimorar o tratamento das dislipidemias, novas estatinas surgiram a partir de modificações químicas da lovastatina, dando origem a sinvastatina e a pravastatina. Outras estatinas consideradas totalmente sintéticas também surgiram, como é o caso da fluvastatina, atorvastatina, rosuvastatina, cerivastatina e pitavastatina (Maron et al., 2000; Mason et al., 2005; Sirtori, 2014). Apesar das diferenças nas propriedades físico-químicas entre as estatinas, todas agem através do mesmo mecanismo de ação, comportando-se como inibidores competitivos da enzima 3-Hidroxi-3-MetilGlutaril Coenzima-A Redutase (HMG-CoA redutase) (Sirtori, 2014).

Desse modo, além da capacidade de reduzir o colesterol, as estatinas apresentam outros efeitos que não podem ser associados apenas às reduções lipídicas, os chamados efeitos não-lipídeo-relacionados ou efeitos pleitrópicos (Liao & Laufs, 2005). Efeitos antioxidantes, ação anticarcinogênica através da inibição da proliferação celular, estabilização de placas ateroscleróticas, efeitos anticoagulantes, melhorias na disfunção endotelial mediada por óxido nitroso, inibição da rejeição de enxertos após transplante de coração e rim, ação no tecido ósseo, efeitos anti-inflamatórios e imunomoduladores, são alguns exemplos de efeitos pleitrópicos proporcionados pelas estatinas (Davignon & Laaksonem, 1999; Bellosta et al., 2000; Blanco-Colio et al., 2003; Liao & Laufs, 2005).

O potencial dessas drogas no controle de respostas infeciosas tem sido bastante explorado (Almog et al., 2006; Gao et al., 2008; Kopterides & Falagas, 2009; Tleyjeh et al., 2009; Janda et al., 2010; Ajroucheet al., 2013; López-Cortés et al., 2013). Esses estudos investigam um possível efeito protetor das estatinas, atuando na redução na morbidade e mortalidade de várias doenças infecciosas. A maioria dos estudos aponta uma redução nas taxas de mortalidade associadas à sepse e bacteremias entre os pacientes que fazem uso das estatinas para a redução de colesterol (Tleyjeh et al., 2009; Janda et al., 2010; Ajroucheet al., 2013; López-Cortés et al., 2013). Entretanto, outros estudos demonstram ausência desse efeito protetor (Wan et al., 2014). Diversos mecanismos que poderiam explicar esse efeito das estatinas na proteção de respostas infecciosas foram propostos, inclusive um efeito antibacteriano (Jerwood & Cohen, 2008; Janda et al., 2010).

A atividade antibacteriana apresentada pelas estatinas tem sido investigada por estudos *in vitro* e *in vivo* (Rego et al. 2007; Horn et al., 2008; Jerwood & Cohen, 2008; Bergman et al., 2011; Masadeh et al., 2012). A sinvastatina se mostrou eficaz contra diversos patógenos como, por exemplo, *Streptococcus pneumoniae*, *Haemophilus influenzae*, *Moraxella catarrhalis* (Bergman et al., 2011), *Escherichia coli*, *Pseudomonas aeruginosa*, dentre outros (Masadeh et al., 2012) Além de apresentar atividade contra *Staphylococcus aureus*, esse fármaco também foi capaz de inibir a invasão celular por esse microrganismo (Horn et al., 2008). Outras estatinas também apresentaram atividade *in vitro* (Masadeh et a., 2012).

Staphylococcus aureus é o principal patógeno associado às bacteremias, representando 40% dos casos. Dentre as cepas mais comumentes associadas a esse tipo de infecção, temos *S. aureus* meticilina-resistente (MRSA) e *S. aureus* oxacilina-resistente (SARO). Outros patógenos que também apresentam alta associação com as bacteremias são: *Pseudomonas aeruginosa*, *S. epidermides*, *Enterobacter* spp, *Escherichia coli*, *Klebsiella* spp e *Acinetobacter* spp (Lark et al., 2000; Sievert et al., 2013).

O gênero *Staphylococcus* possui ampla associação às infecções oportunistas e está presente na microbiota anfibiôntica da pele e nas superfícies mucosas de humanos, principalmente da cavidade nasal**,** axilas, boca, vagina e intestino (Foster et al., 2014). São responsáveis por produzir diversos fatores de virulência, como toxinas, adesinas e componentes de evasão imunológica, que são importantes, principalmente, na interação do microrganismo com o hospedeiro durante o processo inicial de colonização (adesão e invasão), nos mecanismos de evasão (fuga) das defesas do hospedeiro e na modulação da resposta imune (Gill et al., 2005; Foster et al., 2014). Essa característica, somada à grande capacidade que possuem em formar biofilmes, resulta em uma estratégia fácil para a sobrevivência em ambiente hostil, tornando as células bacterianas menos acessíveis ao sistema de defesa do organismo e aos antimicrobianos (Stoodley & Stoodley, 2005). Essa situação acaba dificultando o tratamento de infecções hospitalares, principalmente aquelas oriundas de dispositivos médicos, e essa consequente falha na terapia bacteriana resulta na necessidade de substituição dos dispositivos, aumento do risco dos pacientes e seleção de bactérias resistentes aos antimicrobianos (Mónzon et al., 2002).

Os microrganismos dificilmente são encontrados na forma livre no ambiente; para sobreviver diante das adversidades do ambiente eles crescem em comunidades denominadas biofilmes (Renner & Weibel, 2011). Os biofilmes podem ser definidos como comunidades complexas de microrganismos, aderidos em uma superfície e envoltos por uma matriz extracelular. A formação do biofilme envolve 4 passos principais: (1) adesão a superfície, (2) produção da matriz extracelular, (3) formação de microcolônias e intensificação da produção da matriz extracelular e (4) dispersão do biofilme (Renner & Weibel, 2011; Abdalla et al., 2014). A produção da matriz extracelular é de extrema importância para o processo de maturação do biofilme, sendo composta por proteínas, ácidos nucléicos, lipídeos e principalmente por polissacarídeos. A matriz além de permitir que a adesão inicial dos microrganismos (passo 1) se torne irreversível, auxilia na estrutura do biofilme e acaba criando uma barreira contra estímulos mecânicos e

químicos, promovendo uma maior proteção contra o sistema imunológico e uma maior resistência a antimicrobianos (Renner & Weibel, 2011).

Na busca de novos medicamentos antimicrobianos, uma alternativa interessante é a descoberta de novas funções ou a potencialização das atividades de medicamentos já conhecidos e amplamente utilizados (Fernandes et al., 1999). Este tipo de estratégia mostra-se claramente mais vantajosa tanto em termos de investimentos como em relação ao tempo de execução (Masunari & Tavares, 2006). Nesse contexto, as estatinas aparecem com um grande potencial a ser explorado. Esse grupo de fármacos é amplamente prescrito na medicina, possuindo grande margem de segurança e baixa frequência de efeitos colaterais. Dessa forma, o presente estudo teve como objetivo avaliar o efeito das estatinas sobre o crescimento, viabilidade e formação de biofilme de espécies de microrganismos aeróbios patogênicos

CAPÍTULO 1*

Statins and antimicrobial effects: simvastatin as a potencial drug against *Staphylococcus aureus* biofilm.

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ABSTRACT

Simvastatin is an important lipid-lowering agent, which belongs to the statins. These drugs have other effects called pleiotropic. Several studies have explored a possible protective effect of statins to reduce the morbidity and mortality of various infectious diseases. *Staphylococcus aureus* is considering one of the main pathogens of nosocomial infections; its high ability to form biofilms makes the treatment difficult. The present study evaluated the effects of simvastatin on the growth, viability and biofilm formation of *S. aureus*. The MIC of atorvastatin, pravastatin and simvastatin was evaluated against 10 strains of *S. aureus*, *Pseudomonas aeruginosa*, *Escherichia coli* and *Enterococcus faecalis*. As simvastatin showed activity against *S. aureus*, its effect on cell viability was addressed by time-kill and post-antibiotic effect assays. A possible synergistic effect between simvastatin and vancomycin was observed. In addition, the effect of simvastatin was evaluated against biofilms of *S. aureus*. The MIC of simvastatin against *S. aureus* were 15.65 µg/ml (ATCC 29213) and 31.25 µg/ml (ATCC 33591, 43300, 14458 and 6538). The effect of simvastatin was bactericidal at 4x>MIC and bacteriostatic at MIC concentration. No synergistic effect was found between the simvastatin and vancomycin. However, the results obtained against *S. aureus* biofilms showed that, in addition to inhibiting biofilm formation, simvastatin was also able to act against mature biofilms, reducing cell viability and altering the production of polysaccharides. In conclusion, simvastatin has a great potential to be explored against *S. aureus* biofilm, especially in relation to the discovery of new antimicrobial agents.

Key-words: Statins. *Staphylococcus aureus*. Biofilms.

INTRODUCTION

Simvastatin is a lipophilic drug that belongs to the group of statins. The statins are lipid-lowering agents that are involved in the reduction of cardiovascular

morbidity and mortality.[1] These drugs exhibit a good margin of safety and tolerability with a low frequency of side effects, being the most commonly used agents for the reduction of lipids in patients with elevated cholesterol levels.[2,3] All statins act via the same mechanism of action, as a competitive inhibitor of the enzyme 3-hydroxy-3-methyl-glutaryl-CoenzymeA reductase (HMG-CoA), decreasing the biosynthesis of cholesterol and increasing the removal of circulating Low-Density-Lipoprotein (LDL).[4-6]

Statins have effects other than lipid reduction, called pleiotropic effects,[7] such as anti-inflammatory and immunomodulatory activities.[8-10] Many studies have evaluated the effect of statins in the prevention, morbidity and mortality of various infectious diseases. Some of these studies showed that statins can prevent the establishment of infections or even reduce mortality rates in patients that take statins routinely.[11-13] However, other studies did not find any protective effect of statins against infectious diseases.[14] In patients with bacteremia and sepsis, the use of statins was associated with lower mortality in recent studies.[15-17] Interestingly, some studies have also demonstrated an antimicrobial potential of statins against different bacterial species.[18-21] For example, Simvastatin was able to inhibit host cell invasion[18] and *Staphylococcus aureus* growth.[19,20]. In addition, Atorvastatin, Simvastatin and Rosuvastatin showed activity against several reference bacteria and clinical isolates.[21]

Pseudomonas aeruginosa, *Staphylococcus epidermides*, *Enterobacter* spp, *Escherichia coli*, *Klebsiella* spp, *Acinetobacter* spp and especially *Staphylococcus aureus* are frequently involved with nosocomial infections.[22] S. *aureus*, one of the most important etiological agents of both nosocomial and community-onset infections,[23] produces several virulence factors such as toxins, adhesins and components of immune evasion.[24] These characteristics, combined with the ability that this bacteria has to form biofilms results in an increase of survival in a hostile environment.[25] The attached cell of biofilm produces an extracellular matrix composed of polysaccharides, proteins and DNA.[26] This matrix

makes the bacterial cell less accessible to the defense system of the organism and antimicrobial agents, making the treatment of nosocomial infections difficult.[26]

In the present study, we evaluated the activity of simvastatin, atorvastatin and pravastatin against a range of clinically important pathogens through susceptibility methods. We also tested if vancomycin and simvastatin, that was found to have antimicrobial activity, would act synergistically, by using a microtiter checkerboard method. We further evaluated the effects of simvastatin on *S. aureus* adhesion, biofilm viability and polysaccharides and protein production. These studies demonstrated that simvastatin has a significant activity against *S. aureus* biofilm.

METHODS

Chemicals and Experimental Groups

Atorvastatin (atorvastatin calcium salt trihydrate), pravastatin (pravastatin sodium salt hydrate) and simvastatin (Sigma Chemical Co - Louis, MO, USA) were used for the experiments of Minimum Inhibitory Concentration (MIC). Atorvastatin and simvastatin were dissolved in 100% DMSO, while pravastatin was dissolved in distilled-deionised water. The final concentration of DMSO was 2.5%. Both gentamicin and vancomycin dissolved in deionized water were used as antimicrobial standards.

The groups were: test group (culture medium + bacteria + statin or antimicrobial standard), positive control group (culture medium + bacteria) and vehicle control group (culture medium + bacteria + DMSO). Groups without inoculum but with any of the tested substances were considered as negative controls. All tests were performed using six replicates per group, in two separate occasions at least.

Bacterial strains and Culture Conditions

The following strains were used: *Staphylococcus aureus* ATCC 29213, *S. aureus* ATCC 6538, *S. aureus* ATCC 14458, *S. aureus* methicillin-resistant ATCC 33591, *S. aureus* methicillin-oxacillin-resistant ATCC 43300, *Pseudomonas aeruginosa* ATCC 27853, *P. aeruginosa* ATCC 25619, *Escherichia coli* ATCC 25922, *E. coli* ATCC 10536, *Enterococcus faecalis* ATCC 29212. The bacterial were stored in TSB with 20% of glycerol at - 80 ºC. The strains were routinely cultured on TSA plates, in aerobic conditions, at 35 ^{o}C.

The Muller Hinton Broth (MHB - Difco Co., Detroit, MI, USA) was used for the Minimum Inhibitory Concentration and planctonic tests. For biofilm experiments, the microorganism *S. aureus* ATCC 29213 was cultivated in Brain Heart Infusion (BHI - Difco Co., Detroit, MI, USA) with 1% D-glucose (Sigma Chemical Co - Poole, UK).

For all the following tests, the bacterial inoculum was prepared in 0.9% NaCl considering an optical density of 0.1 at 660 nm, which was equivalent to 1-2 x 10^8 CFU/ml. In each test, the amount of initial bacterial load was 5 x 10^5 CFU/ml.

MIC

MIC was determined by broth microdilution method as described by the Clinical and Laboratory Standards Institute (CLSI).[27] The concentrations for all statins ranged from 250 to 0.24 µg/ml and for antimicrobial standards from 100 to 0.06 µg /ml. Two-fold dilutions were made in 96-well plates with 100 µl of MHB per well. Then, the bacterial suspension (100 µl) was inoculated and the plates were incubated for 24 h at 35 ^{o}C. The lowest concentration with any visible bacterial growth was considered the MIC. In addition, bacterial growth was assessed by optical density measurement (660 nm).

Time-kill assays

For all the following assays, vancomycin and simvastatin, which as the statin whit the best antimicrobial activity, were tested against *S. aureus* ATCC 29213.

The time kill assay was adapted from the method previously described by Eliopoulus & Moellering,[28] and Raja et al.,[29] MIC and 4x>MIC concentrations were chosen according to the data observed in the previous experiments. The time kill assay was performed as described in the MIC assay, and 25-µl samples were taken from the microtiter plates after 0, 2, 4, 8 and 12 h of incubation and spread on TSA plates. The viable colonies were counted after 24 h incubation to determine the CFU/ml. Killing curves were constructed by plotting the $\log_{10}$ CFU/ml versus time over 12 h.

Post-antibiotic effect (PAE)

The determination of post- antibiotic effect (PAE) was adapted from Craig & Gudmundsson,[30] and Raja et al.,[29] The concentrations used were 4x>MIC, 2x>MIC, MIC and ½MIC. 24-well plates containing the tested substances were incubated for 2 h. The samples were centrifuged for 5 minutes at 1400 *g*, the supernatant was removed and new fresh medium was added. This procedure was repeated two times to ensure complete removal of drugs.

The pellet was resuspended in culture medium, diluted 1:10 in tubes containing a final volume of 5 ml of culture medium and incubated at 35 °C. Samples of 25 µl were collected, before and after washing at every hour until visible growth (OD_{660nm} = 0.3), and plated on TSA plates to obtain viable counts. The PAE was calculated using the following equation: PAE = T – C, where T is the time required for the initial bacterial culture increased by 1 $\log_{10}$CFU/ml from the removal of the antimicrobial, and C represents the time required for bacterial cultures not treated with antimicrobial to have an increase of 1 $\log_{10}$ CFU/ml.

Checkerboard Microdilution Assay

In order to evaluate a possible interaction between simvastatin and vancomycin, a checkerboard microdilution assay as described by Odds et al.,[31] and Sun et al.,[32] was used. Simvastatin and vancomycin were prepared at four times of the final concentration in separeted plates. Then, 50 µl simvastatin and 50 µl of vancomycin were mixed and transfered to a new plate. Finnaly, 100 µl bacterial suspension was inoculated and the plates incubated in the same conditions. The analyses of results were based on the value of FICI ("fractional inhibitory concentration index") that was calculated using the following formula:

$$\sum FICI = FICI_A + FICI_B = MIC_{AB}/MIC_A + MIC_{BA}/MIC_B$$

where MIC_A and MIC_B are the MICs of drugs A and B when acting alone and MIC_{AB} and MIC_{BA} are the MICs of drugs A and B when acting in combination, respectively.

FICI values < 0.5 represent synergism in the interaction between drugs. FICI values between 0.5 < FICI < 4.0 are classified as indifferent and where FICI values are > 4.0, they are classified as antagonism.

Biofilm formation assay

The experiments of biofilm formation were carried out using U-bottom 96-well plates. The concentrations for simvastatin and vancomycin ranged from 1/28xMIC to 4x>MIC. This test was conducted similarly to the experiments of MIC, being the substances added at the beginning of biofilm formation (t=0).

After 24 h the incubation, the plates were washed with distilled-deionised water for removing dead or unattached cells. After drying at room temperature, the quantification of biofilm formed in each well was made by optical density (OD_{575nm}) with 0.4% crystal violet solution and 100% ethanol.[33]

Analysis of biofilm formation by scanning electron microscopy (SEM)

The effect of simvastatin on biofilm formation was analyzed by SEM. The biofilm was formed as described in the previous item, with the following modifications. After growing for 24 h in Lab-Tek® Chambers (Nunc, Naperville, IL, USA), glutaraldehyde/PBS 3% (v / v, pH 7.4) were added for 12 h for fixation of samples. Then, the samples were dehydrated with ethanol (50% to 100%). The biofilms were coated with gold and examined with JEOL JSM5600LV (JEOL Ltd., Tokyo, Japan) scanning electron microscopy.[34]

Biofilm viability assay

To assess the effect of simvastatin on the viability of mature biofilm, 24 h-biofilms were exposed to the antimicrobial drugs. Biofilms of *S. aureus* ATCC 29213 were formed in cellulose acetate membranes filters (diameter: 25 mm; pore size: 0.2 µm; Sartorius AG, Germany) placed at the bottom of 6-well plates.

After biofilm formation, the membranes were transferred to a new plate containing culture media and the antimicrobial substances at 4x>MIC. The plates were incubated for 24 h at 35 ^{o}C. The membranes were then washed three times with 0.9% NaCl and sonicated (Vibra Cell 400W, Sonics & Materials Inc., Newtown, CT, USA) with 5% range by 30 seconds for dispersion of the biofilm. Samples of 10 µl were collected, plated on TSA and kept at 35°C under for 24 h, when viable counts were then obtained.

Quantification of polysaccharides, proteins and biomass

In addition to viability of the biofilm, the production of polysaccharides, proteins and biomass (dry weight) after exposure to simvastatin was also analyzed.

The biofilms were formed in the membranes and sonicated as described previously. 800 µl were collected for the extraction of polysaccharides as described by Aires et al.[35] and quantified by the phenolsulfuric method.[36] The protein extraction was carried out using a 2M NaOH solution,[37] and subsequently quantified by colorimetric assays through BCA protein quantification (Thermo scientific, Scottdale, AZ, USA). For biomass (dry weight), 1000 µl of each sample were centrifuged, and the pellet was dried in a lyophilizer (Lyo Chamber Guard Christ LCG 121505 PMMA (Nova Analítica) Alpha 2-4 LD plus) e then weighed.

Statistics

All tests were performed using six replicates per group, in two separate occasions at least. Data was analyzed by using GraphPad version 5.00 (San Diego, California, USA). The normality of data was tested by using Shapiro-Wilks test. Data with normal distribution were compared using an ANOVA, and significant differences between control and treatment groups were determined using the Bonferroni *post-hoc* test. Data abnormally distributed were analyzed by Kruskal-Wallis and Dunn *post-hoc* tests. The significant level was set at 5%.

RESULTS

Simvastatin but not Atorvastatin and Pravastatin has antibacterial activity against *S. aureus*

Simvastatin showed activity against all strains of *S. aureus*, but had no effect against the other species tested. The MIC values for simvastatin and the antimicrobial standards are shown in Table 1. No MICs for atorvastatin and pravastatin were observed at the concentrations tested. The concentrations of DMSO (2.5% V/V) used in all tests did not interfere with bacterial growth. The

strains *S. aureus* 29213, 33591, 14458, 43300 and 6538 and *E. faecalis* were sensitivity to vancomycin, while for gentamicin only *S. aureus* 43300 was resistant. All values of MIC for the antibiotics were in accordance to CLSI.

Table1. Minimum Inhibitory Concentration (µg/ml) for simvastatin, gentamicin and vancomycin.

Bacterial strains	**Simvastatin**	**Gentamicin**	**Vancomycin**
S. aureus 29213	15.65	0.78	1.56
S. aureus 33591 (MRSA)	31.25	3.12	1.56
S. aureus 14458 (ORSA)	31.25	0.78	1.56
S. aureus 43300	31.25	----	1.56
S. aureus 6538	31.25	1.56	1.56
P. aeruginosa 27853	----	0.78	----
P. aeruginosa 25619	----	0.39	----
E. coli 25922	----	1.56	----
E. coli 10536	----	0.78	----
E. faecalis 29212	----	6.25	3.12

Statins effect on *S. aureus* strains are dose and drug dependent, as observed in Figure 1. Pravastatin also showed a reduction on *S. aureus* strains growth, but it did not completely inhibit these strains. Simvastatin effects against *S. aureus* were more prominent, even considering sub-MIC concentrations ($p < 0.05$).

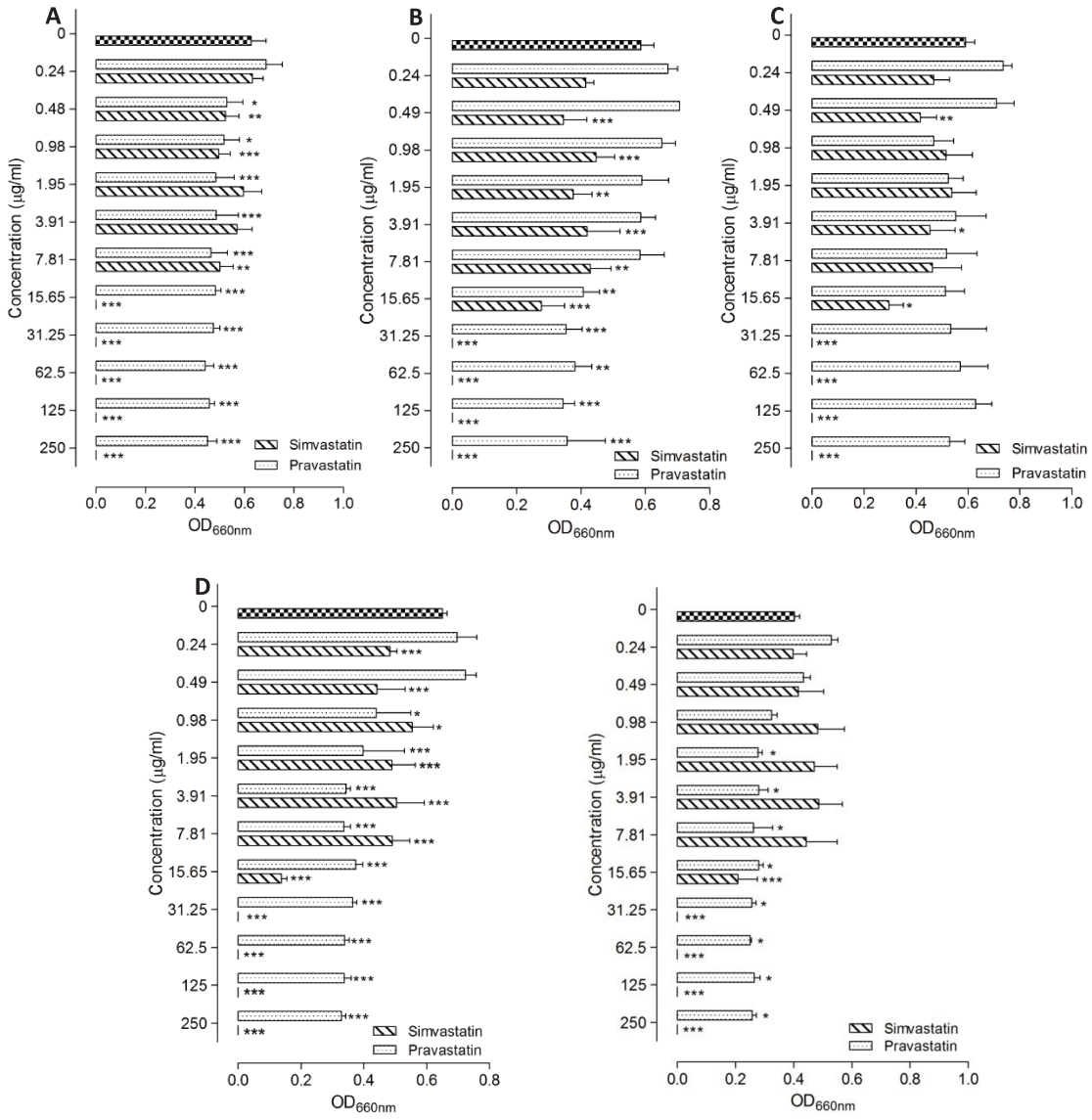

Figure 1. Mean and standard deviation of optical density (660 nm) representing the bacterial growth of *S. aureus* when exposed to simvastatin and pravastatin. **A.** *S. aureus* 29213. **B**. *S. aureus* MRSA 33591. **C**. *S. aureus* MRSA 43300. **D**. *S. aureus* 14458. **E**. *S. aureus* 6538. Significant differences between the treatment and the control group were considered when *p<0.05, **p<0.01 or ***p<0.001 (2-way ANOVA, Bonferroni).

Figure 2 shows the effect of simvastatin on *S. aureus* cell viability during 12 h of exposure. Simvastatin at a concentration of 4x>MIC exhibited a bactericidal effect against *S. aureus* ATCC 29213, causing a reduction in the number of viable cells (Fig. 2A), while the MIC concentration showed a bacteriostatic effect (Fig. 2B), since the number of cells remained constant during 12 h. Vancomycin showed a bactericidal effect for both concentrations studied. However, more time was required to kill 100% of cells for the lower concentration.

Figure 2C shows the post-antibiotic effect (PAE) for both drugs. Vancomycin showed greater PAE than Simvastatin; however, no differences ($p > 0.05$) between the PAE of the two drugs was observed at 4x>MIC. DMSO did not showed any effect in both time-kill and PAE assays.

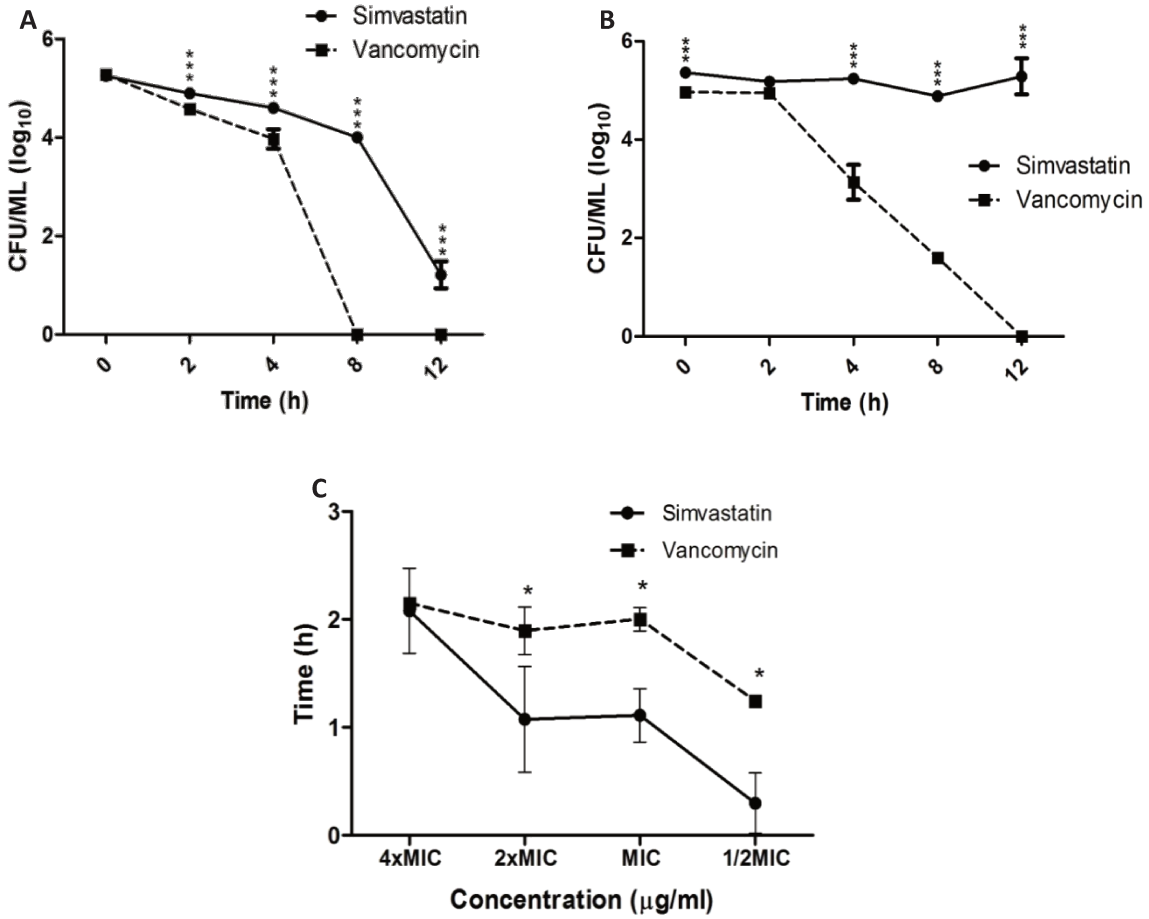

Figure 2. **A**. Effect of simvastatin and vancomycin on cell viability during 12 h exposure, concentration equivalent to 4xMIC. **B**. Effect of simvastatin and vancomycin on cell viability during 12 h exposure, concentration equivalent to MIC. **C**. Post-antibiotic effect of simvastatin and vancomycin. Significant differences between the treatment and the control group when *p<0.05 or ***p<0.001 (ANOVA 2way).

Simvastatin has no synergistic effect with Vancomycin

FICI values were higher than 0.5 as shown in Table 2, indicating that there is no synergic effect between simvastatin and vancomycin against *S. aureus*.

Table 2. FICI[1] values for association between simvastatin and vancomycin. FICI < 0.5 = synergism. 0.5 < FICI < 4.0 = indifferent. FICI > 4.0 = antagonism.

Bacterial strain	FICI
S. aureus 29213	0.56
S. aureus 33591	1.06
S. aureus 14458	1.00
S. aureus 43300	1.06
S. aureus 6538	1.03

Simvastatin inhibits biofilm formation of *S. aureus*

Figure 3A shows the absorbance values for simvastatin and antimicrobial standards. The concentrations tested ranged from 1/128 MIC to 4x MIC, thus the concentrations for each drug were: 0.12 - 62.6 µg/ml for simvastatin and 0.012 - 6.24 µg/ml for vancomycin.

Simvastatin from 1/8 MIC up to 4x MIC (1.95 to 62.6 µg/ml) reduced significantly the biofilm formation ($p < 0.05$). Inhibition of biofilm was observed for the antimicrobial standard drug at 1/2MIC (0.78 µg/ml). When analyzed by MIC range, simvastatin could reduce biofilm formation more significantly until the concentration 1/64 MIC when compared to vancomycin ($p < 0.005$). DMSO did not alter *S. aureus* ATCC 29213 biofilm formation ($p > 0.05$). The images obtained by SEM confirmed the inhibitory effect of Simvastatin on *S. aureus* 29213 biofilm (Figure 3B).

[1] FICI: fractional inhibitory concentration index

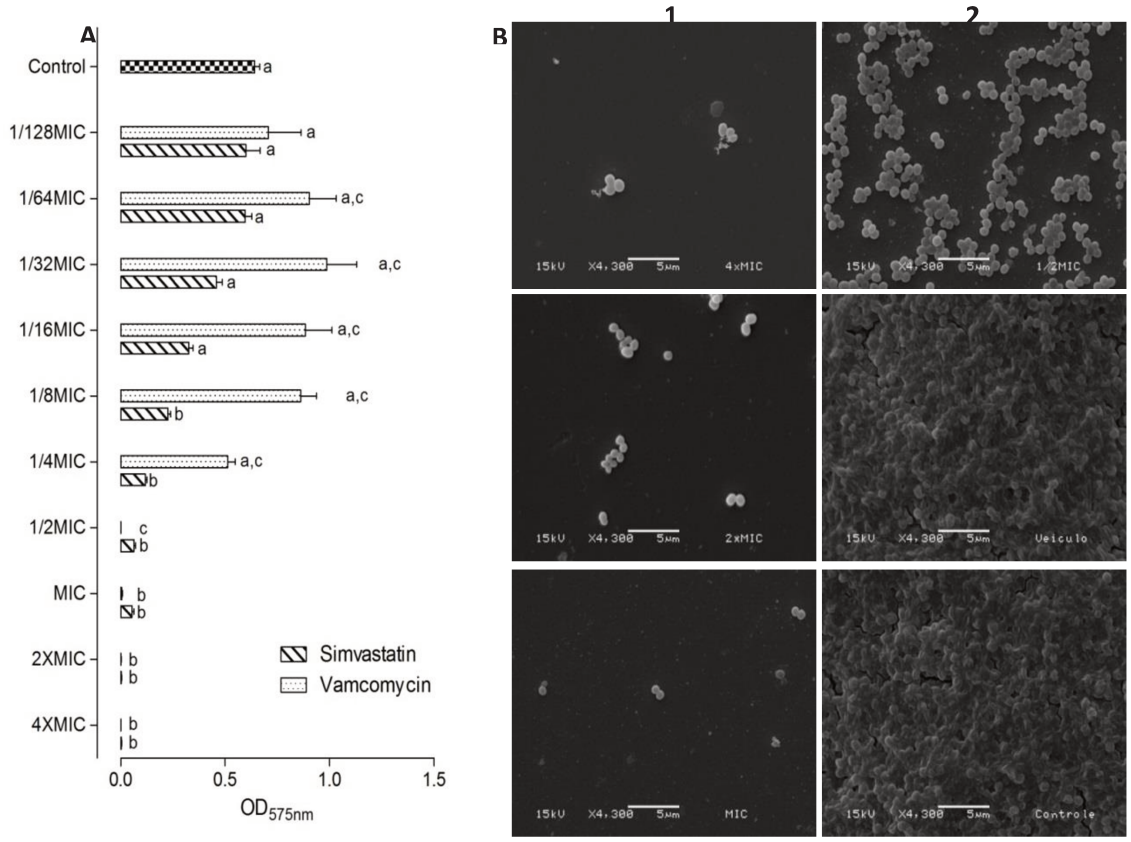

Figure 3. A. Mean and standard deviation of absorbance (OD_{575nm}) representing the biofilm formation of *S. aureus* 29213 in the presence of simvastatin e vancomycin. Different letters represent statistical difference; comparisons were made at each dilution and the control group (Kruskal-Wallis). **B**. Images obtained by SEM representing the biofilm formation of *S. aureus* 29213 in the presence of Simvastatin. In column 1 are the 4x MIC, 2X MIC and MIC concentrations, respectively. In column 2 are the concentration ½ MIC, vehicle group (DMSO) and the control group, respectively.

Simvastatin decreases cell viability and alters the production of polysaccharides in mature biofilms

The cell viability of *S. aureus* 29213 after treatment with simvastatin and vancomycin is exposed in Figure 4. The results showed that simvastatin (4x MIC = 62.6 µg/ml) could reduce significantly viable cells of biofilm when compared to the control and vehicle group ($p<0.005$), while vancomycin (4x MIC = 6.24 µg/ml) showed no difference when compared to group control ($p > 0.05$). DMSO did not reduce the number of viable cells. Thus, simvastatin was effective against mature biofilm of *S. aureus* 29213, and despite the concentrations of the statin and antibiotic are different, the proportion in relation to the MIC was the same for all drugs.

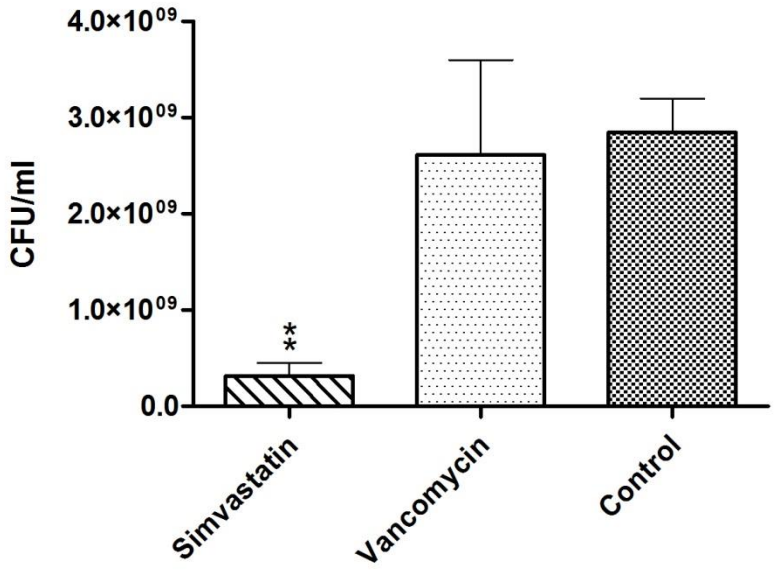

Figure 4. Mean and standard deviation of CFU/ml representing the cell viability of *S. aureus* 29213 biofilm after exposed to simvastatin and vancomycin. Significant differences between the treatment and the control group when $**p < 0.01$ (Kruskal-Wallis).

As simvastatin inhibited *S. aureus* ATCC 29213 biofilms, it is possible that this statin interfered in the extracellular matrix of biofilm. The production of extracellular polysaccharide soluble (EPS) was very low (data not shown). The results for extracellular polysaccharide insoluble (EPSI) and intracellular

polysaccharide (IPS) are both shown in Table 3. Simvastatin reduced the production of EPSI ($p < 0.05$) and increased the production of IPS when compared with control ($p < 0.05$). However, simvastatin did not change the total proteins production ($p > 0.05$). DMSO did not alter the amounts of polysaccharides, proteins and biomass.

Table 3. Effects of simvastatin on the production of polysaccharides, proteins and biomass of *S. aureus* biofilm. [2]

	Polysaccharides (µg/mg dry weight)		Proteins (µg/mg dry weight)	Biomass (mg)
	EPSI	IPS		
Simvastatin	22.7 ± 9.0*	78.6 ± 25.4*	2.3 ± 0.4	0.66 ± 0.12*
Control	40.7 ± 9.6	34.1 ± 8.6	1.9 ± 0.4	0.93 ± 0.13

DISCUSSION

The potential of pleiotropic effects exhibited by statins conducted a series of studies to investigate the role of these drugs in the development of infections.[15-17,38,39] The antimicrobial activity of these drugs was proposed and investigated by some authors.[18-21] We evaluated the antimicrobial activity of atorvastatin, pravastatin and simvastatin against 10 bacterial strains associated with nosocomial infections. In addition, as simvastatin showed activity against *S.*

[2] The values of polysaccharides and proteins were normalized by dry weight. Significant differences between the treatment and the control group when *$p < 0.05$ (Kruskal-wallis).

aureus, to obtain more information on antibacterial activity of this drug, we explored its effects on planktonic cells and biofilm of *S. aureus* 29213.

In our study, simvastatin showed 100% of inhibition only against *S. aureus*. In addition, sub-MIC concentrations were able to reduce the growth of *S. aureus* even at concentrations lower than MIC (0.24 µg/ml). The MICs found in the present study were lower than the MICs showed previously in other studies considering *S. aureus,*[20] and other bacterial species.[21] However, the values found for antimicrobial standards are in accordance to CLSI,[27] demonstrating that the method used in our study was suitable. Finally, Bergman et al.,[19] found a MIC value of 15.6 µg/ml for simvastatin against *Streptococcus pneumoniae*, which is a concentration very close to the ones found in the present study.

Atorvastatin and pravastatin did not present full inhibitory activity, as demonstrated by the MIC tests. However, pravastatin, and also simvastatin were able to reduce the growth of *S. aureus, E. coli*, *P. aeruginosa* and *E. faecalis* (data not shown). Masadeh et al.,[21] reported inhibitory activity for simvastatin and atorvastatin against several species, including *E. coli*, *P. aeruginosa* and *E. faecalis.* The enhanced antimicrobial activity of simvastatin in comparison to pravastatin and atorvastatin may be related to differences in their chemical characteristics, as described previously.[20,21]

Pravastatin and simvastatin are semi-synthetic forms, derivatives of lovastatin, a metabolic product of *Penicillium citrinum*,[40] being atorvastatin the total synthetic form.[2] Simvastatin and atorvastatin are lipophilic, while pravastatin has hydrophilic properties.[41] Thus, simvastatin probably cross the cell membrane more easily, causing bacterial inhibition in a dose dependent manner. Although lipophilic, atorvastatin has no significant antimicrobial activity. This molecule is not derived from a fungal metabolite, and these would be the reason for lacking antimicrobial effects. However, further studies on structure-activity relationship should be carried out to better understand the antimicrobial properties of statins.

To better understand the antimicrobial properties of statins, we investigated its effects on *S. aureus* 29213 in planktonic and biofilm assays, since this strain has a better ability to form biofilm when compared with resistant strains. We first evaluated the cell viability when *S. aureus* 29213 was exposed to simvastatin for 2, 4, 8 and 12 (time kill assays). At 4x MIC, simvastatin reduced the number of viable cells, especially after 12 h of exposure.But, at MIC concentration, the number of viable cells remained constant during all periods of exposure. . Thus, while the 4x MIC killed *S. aureus* 29213, the MIC only inhibited the growth, thus demonstrating that the type of effect exhibited by simvastatin, bactericidal or bacteriostatic, is dose-dependent. However, vancomycin showed a bactericidal effect for both concentrations, reducing the number of viable cells more significantly than simvastatin. In addition, we also verified how long these effects persist after the removal of the drug, also known as post-antibiotic effect (PAE). For concentration 4x MIC, simvastatin and vancomycin showed a similar PAE. This find emphasizes that the effect is due to prior antimicrobial properties rather than to persisting sub-inhibitory concentrations.

The ability of simvastatin to produce PAE similarly to vancomycin is an interesting finding, as theoretically it could suppress bacterial growth even when concentrations fall below the MIC.[30]

Previous studies reported *in vitro* synergistic and antagonistic effects of the association among statins and antifungals.[42-44] Therefore, we investigated a possible interaction between simvastatin and vancomycin by checkerboard test. However, the combination of these drugs had no synergistic effect against any strain of *S. aureus*. The FICI value found for *S. aureus* 29213 was low compared to the other strains, but more studies are needed to verify if this interaction has some potential.

Despite the importance of determining the MIC and the antibacterial activity against planktonic cells, microorganisms are not usually found in body-liquids.[45] To face the adversities of the environment, microorganisms adhere on surfaces and grow grouped involved by extracellular polymeric substances (EPS),

forming communities known as biofilms.[45,46] Biofilm formation involves four steps: (1) reversible adhesion to a surface, (2) production of EPS (irreversible adhesion), (3) microcolonies formation and intense production of EPS and (4) biofilm dispersion.[46,47]

Our results show that simvastatin was able to inhibit biofilm formation in concentrations lower than the MIC (8x < MIC). This inhibition was confirmed by images obtained by scanning electron microscopy (SEM). Considering both vehicle and control groups, it was possible to observe a biofilm in a mature stage, with the cells immersed in an extracellular matrix. In the presence of simvastatin, however, few cells are adhered on the surface considering drug-concentrations equal or higher than MIC. For concentration ½ MIC, more cells were adhered, but biofilm failed to develop and achieve step 3. Thereby, simvastatin inhibits biofilm formation by *S. aureus*, probably preventing adherence of cell in concentrations higher than MIC and the development of biofilm in sub-MIC concentrations.

To fight mature biofilms has become a challenge. They are more resistant to antimicrobial agents, which difficult the treatment, leading to complications for the patient.[48] We investigated the effect of simvastatin against *S. aureus* biofilms with 24 h of growth, in an more advanced stage of maturation. Simvastatin exhibited excellent activity in concentrations at 4x MIC, reducing the cell viability at 10x, while vancomycin exhibited no effects. The concentration of antibiotic required to kill cells in biofilm is much higher than to kill planktonic cells, sometimes 100 or 1000 times the MIC.[49] Therefore, our findings revealed a potential of simvastatin to be explored, since in concentrations only 4 times the MIC they have had an excellent ability to decrease cell viability. Unfortunately, the difficult dilution of simvastatin did not allow testing higher concentrations.

Several mechanisms explaining the resistant of biofilms have been described.[48,49] The extracellular matrix is implicated as an important mechanism, especially by decreasing the penetration of an antibiotic.[49] We hypothesized that effect of simvastatin could also involve an effect on two important components of EPS, polysaccharides and proteins. The EPSI contribute to structure, being

responsible for integrity of biofilms.[45,50] Some EPSI are also associated with resistance in bacterial biofilms, as the Poly-(1,6)-*N*-acetyl-D-glucosamine (PNAG), the major extracellular polysaccharide in *S. aureus*, is responsible for preventing fluid convection and the transport of solute through biofilms.[51] After treatment with simvastatin, the biofilm showed a reduction in the production of EPSI and an increase in the production of IPS, when compared to the control group. The increase in production of IPS in some bacteria such as *S. mutans* is associated to a nutrition reservoir allowing to extent the survival in limiting conditions.[52] A possible hypothesis for this finding would be that in attempt to increase its survival in presence of simvastatin, *S. aureus* decreases the production of EPSI to produce reserve polysaccharides. This change could also explain the excellent effect of simvastatin on the *S. aureus* viability.

Since EPSI is responsible for biofilm structure and it is a major component of the extracellular matrix, a reduction in its production could lead biofilms more accessible to drugs. However, more studies are needed to understand the role of simvastatin in the production of polysaccharides.

Simvastatin did not alter significantly the amount of proteins. The production of proteins is generally more intense during biofilm formation, having a key role in the colonization of biofilm.[50] Thus, the absence of effect on the protein production is understandable. Perhaps a qualitative study can better respond if simvastatin has some effect on the production of proteins. The reduction in biomass of biofilm is probably due to reduction of the polysaccharides, since they are the major fraction of the EPS.[45]

The concentrations found to have antimicrobial properties are a thousand times higher than the plasmatic concentrations raised in patients under statins therapy.[19] However, regardless of whether the physiological concentrations of simvastatin present or not bacterial activity, our results highlight an antimicrobial potential to be explored. Our study was the first to investigate the effect of simvastatin on bacterial biofilms, showing a great antimicrobial activity for this statin. The identification and development of new antibiotics, especially those with

new mechanism of action, are of most importance in public health worldwide.[53] We believe that studies on its molecular structure would bring a new antibacterial pharmacophore, which would be useful in the future as a template to the development of new antibiotics.

In conclusion, simvastatin has antimicrobial activity against *S. aureus* biofilm, reducing its formation, viability and polysaccharides production. These findings can contribute to the search for new antibacterial drugs, considering the potential of simvastatin as an antibiotic prototype.

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CONCLUSÃO

Os resultados demonstram que a sinvastatina apresentou atividade contra *S. aureus*, sendo capaz de impedir a formação de biofilme, e agir contra biofilmes já formados, diminuindo a viabilidade celular e alterando a produção de polissacarídeos. Em conclusão, essa droga apresentou um excelente potencial a ser explorado para obtenção de novos antimicrobianos.

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